Modification of transcription factor Zbtb21 by SUMO (#86140)

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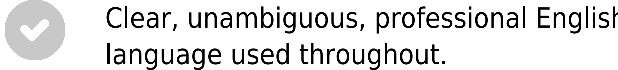

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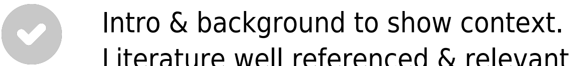

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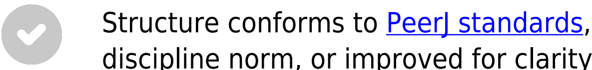

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Modification of transcription factor Zbtb21 by SUMO

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Background. Post-translational modification by SUMO (S mall U biquitin-like MO difier) is an important mechanism to regulate the activity of transcription factors. Zbtb21 is a zinc finger and BTB (B road-complex, T ram-track and B ric à brac) domain-containing transcription factor. Bioinformatic prediction suggests several putative SUMOylated sites in Zbtb21 protein.

Methods. Two evolutionarily conserved lysine residues in Z btb 21 protein were mutated alone or in combination to abolish the binding with SUMO molecules. Western blot and co-immunoprecipitation analyses were performed to detect the SUMOylation state of wild type and mutant Zbtb21 proteins, respectively. Luciferase reporter assays were conducted to evaluate their transcription activities. Meanwhile, immunofluorescence staining was carried out to show their sub-nuclear localizations. Finally, co-immunoprecipitation was performed to detect the interaction between Zbtb21 and its partners.

Results. Phylogenetically conservedlysines 419 and 845 of zebrafish Zbtb21 protein can be conjugated with SUMO molecules. SUMOylation does not affect the subcellular localization and protein stability of Zbtb21, as well as the interaction with Zbtb14 or Zbtb21. Nevertheless, luciferase reporter assays revealed that Zbtb21 is a dual-function transcription factor which exerts activation or repression effect on different promoters, and SUMOylation can modulate the transcriptional activity of Zbtb21 in regulating downstream target genes. Hence, Zbtb21 is identified as a novel substrate of SUMOylation, which would be important for its function.

1 **Modification of transcription factor Zbtb21 by SUMO**

2

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15 **Abstract**

16 **Background.** Post-translational modification by SUMO (Small Ubiquitin-like MOdifier) is an
17 important mechanism to regulate the activity of transcription factors. Zbtb21 is a zinc finger and
18 BTB (Broad-complex, Tram-track and Bric à brac) domain-containing transcription factor.
19 Bioinformatic prediction suggests several putative SUMOylated sites in Zbtb21 protein.
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21 **Methods.** Two evolutionarily conserved lysine residues in Zbtb21 protein were mutated alone or
22 in combination to abolish the binding with SUMO molecules. Western blot and co-
23 immunoprecipitation analyses were performed to detect the SUMOylation state of wild type and
24 mutant Zbtb21 proteins, respectively. Luciferase reporter assays were conducted to evaluate their
25 transcription activities. Meanwhile, immunofluorescence staining was carried out to show their
26 sub-nuclear localizations. Finally, co-immunoprecipitation was performed to detect the
27 interaction between Zbtb21 and its partners.
28
29 **Results.** Phylogenetically conserved lysines 419 and 845 of zebrafish Zbtb21 protein can be
30 conjugated with SUMO molecules. SUMOylation does not affect the subcellular localization and
31 protein stability of Zbtb21, as well as the interaction with Zbtb14 or Zbtb21. Nevertheless,
32 luciferase reporter assays revealed that Zbtb21 is a dual-function transcription factor which
33 exerts activation or repression effect on different promoters, and SUMOylation can modulate the
34 transcriptional activity of Zbtb21 in regulating downstream target genes. Hence, Zbtb21 is
35 identified as a novel substrate of SUMOylation, which would be important for its function.

37 **Introduction**

38 Post-translational modification by SUMO (Small Ubiquitin-like MOdifier) is an important
39 mechanism to regulate many biological processes such as embryogenesis, hematopoiesis, and
40 tumorigenesis. The process of protein SUMOylation is accomplished by a sequential cascade
41 involving E1 activating enzymes (SAE1/SAE2), E2 conjugating enzyme (UBC9), and E3
42 ligases, which transfers the SUMO molecules to target lysine residues in specific substrates
43 (Eifler & Vertegaal 2015). SUMO1, SUMO2, and SUMO3 are three major SUMO paralogs in
44 mammals. While SUMO1 protein shares only approximately 46% identity to SUMO2/3,
45 SUMO2 and SUMO3 are highly similar(Eifler & Vertegaal 2015). SUMOylated lysines of a
46 substrate protein are frequently located within the consensus sequence ΨKXE (where Ψ is a
47 hydrophobic residue) (SampsonWang M Fau - Matunis & Matunis 2001). Unlike SUMO1,
48 SUMO2/3 contain a conserved consensus SUMOylation site at the N-terminus, indicating
49 SUMO2/3 are capable to form poly-SUMO chains (TathamJaffray E Fau - VaughanVaughan Oa
50 Fau - DesterroDesterro Jm Fau - BottingBotting Ch Fau - NaismithNaismith Jh Fau - Hay & Hay
51 2001).

52 Accumulating data indicate transcription factors are a major group of SUMO substrates. In
53 most cases described to date, SUMOylation of transcription factors leads to transcriptional
54 repression (Garcia-Dominguez & Reyes 2009; Valin & Gill 2007). Nevertheless, transcriptional
55 activity of some transcription factors is up-regulated upon SUMOylation (KishiNakamura T Fau
56 - NishioNishio Y Fau - MaegawaMaegawa H Fau - Kashiwagi & Kashiwagi 2003). In addition,
57 SUMOylation also plays various roles on the localization, stability, and protein-protein
58 interaction of transcription factors in a substrate-specific manner, which must be addressed
59 experimentally in each individual case (OuyangValin A Fau - Gill & Gill 2009).

60 There are 49 zinc finger and BTB (Broad-complex, Tram-track and Bric à brac) domain-
61 containing transcription factors in human genome. While the consecutive zinc finger motifs at
62 the C-terminus enable the binding with DNA, the BTB motif at the N-terminus mediates the
63 homo/hetero-dimerization/multimerization between different ZBTB proteins (Maeda 2016).

64 Most of ZBTB family members function as transcription repressors through recruiting nuclear
65 corepressors such as SMRT and NCoR (Maeda 2016).
66 It is worth noting that SUMOylation has important influences on the functions of multiple
67 ZBTB transcription factors. For example, ZBTB16 (also named as PLZF) can be modified with
68 SUMO1 on lysines 242, 387 and 396 in the linker region (ChaoChang Cc Fau - Shih & Shih
69 2007), which eventually affects its DNA binding capacity or protein stability; ZBTB29 (also
70 named as HIC1) SUMOylation on lysine 314 in the central region favors its interaction with
71 MTA1 (DehennautLoison I Fau - DubuissezDubuissez M Fau - NassourNassour J Fau -
72 AbbadieAbbadie C Fau - Leprince & Leprince 2013); SUMOylation of ZBTB1 on lysines 265
73 and 328 in the linker region affects its subcellular localization and the binding with SMRT
74 (MaticSchimmel J Fau - HendriksHendriks Ia Fau - van Santenvan Santen Ma Fau - van de
75 Rijkevan de Rijke F Fau - van Damvan Dam H Fau - GnadGnad F Fau - MannMann M Fau -
76 Vertegaal & Vertegaal 2010). In addition, ZBTB2, ZBTB9, and ZBTB38 proteins can also be
77 SUMOylated (Matic et al. 2010).

78 In a recent study, we have identified zebrafish Zbtb14 is a SUMOylated substrate
79 (DengWangLiuYuanXude ThéZhou & Zhu 2022). SUMOylation of Zbtb14 occurs on a
80 conserved lysine residue within the BTB domain, which renders Zbtb14 capable of repressing
81 *pu.1* promoter during zebrafish macrophage development (Deng et al. 2022). We noticed that
82 ZBTB21 was described as a direct interactant with ZBTB14 in HEK293T cells, and ZBTB21
83 could inhibit multiple genes’ promoter (WangKudoh J Fau - TakayanagiTakayanagi A Fau -
84 Shimizu & Shimizu 2005). Moreover, bioinformatic prediction suggested there are several
85 putative SUMOylated sites in ZBTB21 protein. Therefore, we speculated that ZBTB21 would be
86 a potential SUMOylated substrate.

87 Here, we demonstrate that zebrafish Zbtb21 is a novel substrate of SUMOylation,
88 phylogenetically conserved lysines 419 and 845 of Zbtb21 protein can be conjugated with
89 SUMO molecules. SUMOylation does not affect the subcellular localization and stability of

90 Zbtb21, as well as the interaction with Zbtb14 or Zbtb21. Nevertheless, luciferase reporter assays
91 reveal that Zbtb21 is a dual-function transcription factor which exerts activation or repression
92 effect on different promoters, and SUMOylation can modulate the transcriptional activity of
93 Zbtb21 in regulating downstream target genes.

94

95 Materials & Methods

96 Plasmid construction

97 Zebrafish *zbtb21* gene and its serial SUMOylation mutants were cloned into PCS2$^+$ vector.
98 For the luciferase reporters, the –1.5 kb promoter of *CDC6* gene and the –1.1 kb promoter
99 of *zbtb14* gene were cloned into the PGL3 basic vector (Promega, Madison, WI). Primers used
100 were listed in Table 1.

101

102 Cell culture, transient transfection, luciferase reporter assay, and immunofluorescence

103 HEK293T was obtained from ATCC, and the cell line was tested negative for mycoplasma.
104 HEK293T cells were maintained in DMEM (Gibco, Life Technologies, Carlsbad, CA) with 10%
105 fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA). Plasmid transfection was carried
106 out with Effectene Transfection Reagent (Qiagen, Manchester, UK) according to the
107 manufacturer's instruction.

108 For the luciferase reporter assay, HEK293T cells were harvested 48 hours after transfection
109 and analyzed using the Dual Luciferase Reporter Assay Kit (Promega, Maddison, WI), according
110 to the manufacturer's protocols.

111 For microscopic analyses, HEK293T cells were seeded on micro cover glasses in wells of
112 six-well plates 24 hours before transfection. 48 hours after transfection, cells were
113 immunostained with rabbit anti-HA monoclonal (Cell Signaling Technology) at 1:1000 dilution,
114 and visualized with secondary antibody, Alexa Fluor 594 goat anti-rabbit IgG (Cell Signaling
115 Technology), at 1:1000 dilution. Cell nuclei were stained by DAPI (1.0 mg/ml, BD Biosciences)
116 for 1 minute. Fluorescent images were obtained by ECLIPSE E800 microscope (Nikon).
117

118 **Western blot and co-immunoprecipitation assays**

119 HEK293T cells were transfected with indicated plasmids. After 48 hours of transfection, the
120 cells were washed with phosphate-buffered saline (PBS) buffer for 1 minute 3 times. Lysates
121 were prepared using RIPA lysis buffer (Beyotime, Shanghai, China) with proteinase inhibitor
122 (Roche, Basel, Switzerland). After shaking on ice for 30 minutes, the cells were harvested and
123 centrifuged at 15,000 ×g for 30 min at 4 °C. Rabbit anti-HA antibody (Cell Signaling
124 Technology) was mixed with the protein-G-agarose beads (30 μl) in the supernatant at 4°C
125 overnight. The beads were prepared by centrifugation and washed three times with RIPA lysis
126 buffer. Proteins binding to the beads were eluted by adding 30 μl of 2 × SDS sample buffer and
127 analyzed by immunoblotting using anti-SUMO1 (Cell Signaling Technology) or anti-FLAG
128 antibody (Sigma).

129

130 **Statistical analysis**

131 Data were analyzed by SPSS software (version 20) using two-tailed Student's t test for
132 comparisons between two groups and one-way analysis of variance (ANOVA) among multiple
133 groups. Differences were considered significant at $p < 0.05$. Data are expressed as mean ±
134 standard error of the mean (SEM).

135

136 **Results**

137 **Zbtb21 is identified as a SUMOylated substrate**

138 Bioinformatic prediction suggested that K419 and K845 would be two potential SUMOyated
139 lysine residues of Zbtb21 protein, which are located within the linker and zinc finer regions,
140 respectively (SUMOsp2.0 prediction software). To determine whether Zbtb21 is a real
141 SUMOyated substrate, a series of mutants including $Zbtb21^{K419R}$, $Zbtb21^{K845R}$, and
142 $Zbtb21^{K419/845R}$ (lysine was mutated to arginine to abolish the attachment with SUMO
143 conjugates) was constructed and transfected into HEK293T cells. Subsequent western blot
144 analyses revealed that three SUMO adducts could be found above the unmodified Zbtb21
145 protein, which became more intensive in the presence of UBC9 and SUMO1 (Figure 1A, lane 2
146 and 3). Yet, certain SUMO adduct disappeared for $Zbtb21^{K419R}$ and $Zbtb21^{K845R}$ mutant proteins,
147 respectively (Figure 1A, lane 4-7), and no adducts could be observed for $Zbtb21^{K419/845R}$ double
148 mutant (Figure 1A, lane 8 and 9). These results from western blot analyses were further
149 confirmed by immuno-coprecipitation (Co-IP) experiments (Figure 1B).

150 Taken together, these observations suggest Zbtb21 is an authentic SUMOyated substrate, in
151 which K419 and K845 are two SUMO conjugated lysine residues. In addition, it seems that the
152 stability of Zbtb21 protein is not affected upon loss of SUMOylation.

153

154 **SUMOylation of Zbtb21 affects its transcriptional activity**

155 Wang et al demonstrated that ZBTB21 exerted transcriptional repression on *CDC6* gene's
156 promoter (Deng et al. 2022). We therefore carried out luciferase analyses on *CDC6* reporter with
157 wild type Zbtb21 and SUMO-defective mutants, respectively. In addition, *zbtb14*, whose
158 upstream regulatory region could be bound by Zbtb21, was also used for luciferase assay.

159 The results showed that while Zbtb21 displayed a significant repression effect on *CDC6*
160 promoter, it could activate *zbtb14* reporter (Figure 2), suggesting Zbtb21 is a dual function

161 transcription factor whose activity would be context-dependent. Moreover, it is worth noting that
162 both the repression and the activation activity of Zbtb21 were impaired upon loss of
163 SUMOylation (Figure 2).
164 Overall, these results indicate SUMOylation is required for regulation of the function of
165 Zbtb21.
166

167 **SUMOylation of Zbtb21 does not affect its subcellular localization**

168 The nuclear localization signal (NLS) of Zbtb21 is close to K419 SUMOylation site. To
169 address whether Zbtb21 SUMOylation is associated with its subcellular localization,
170 immunofluorescence staining was performed. The results showed that HA-tagged wild type
171 Zbtb21 was localized in the nucleus of HEK293T cells, and similar localization was found for
172 $Zbtb21^{K419R}$, $Zbtb21^{K845R}$, and $Zbtb21^{K419/845R}$ mutants (Figure 3). These observations suggest
173 SUMOylation of Zbtb21 is irrelevant with its nuclear distribution.

174

175 **SUMOylation of Zbtb21 does not affect its interaction with its partners**

176 The interaction between ZBTB14 and ZBTB21 is mediated by both BTB and zinc finger
177 domains (Deng et al. 2022). Since K845 site of ZBTB21 is located within the zinc finger region,
178 we set out to explore whether K845 SUMOylation could affect its binding with ZBTB14. The
179 results from Co-IP assays indicated that the ZBTB21 SUMO mutants could still interplay with
180 ZBTB14 (Figure 4A). Besides, SUMOylation did not affect the homodimer formation of
181 ZBTB21, either (Figure 4B).

182

183 **Discussion**

184 In the current study, we identify that zebrafish Zbtb21 is a novel substrate of protein
185 SUMOylation, of which lysines 419 and 845 are two SUMOylated sites. As many transcription
186 factors reported to date, SUMOylation is also tightly linked with the transcription activity of
187 Zbtb21. Note that the two lysines are conserved across species, implying modification with
188 SUMO is well-conserved during evolution. Actually, once the two lysines were mutated in
189 human ZBTB21, the SUMO adducts also disappeared (data not shown).

190 There are two isoforms for human ZBTB21: ZBTB21L and ZBTB21S. It is worth noting
191 that the two lysines which can be conjugated with SUMO molecules are both retained in the long
192 and short isoforms, implying SUMOyation would be important for the function of each ZBTB21
193 isoform.

194 Three major types of SUMOs (SUMO1 and SUMO2/3) exist in vertebrates. Amongst these
195 paralogs SUMO1 usually modifies its substrates as a monomer (an 11 kD peptide post-
196 translationally attached to target lysine residues), yet, SUMO2/3 can form poly-SUMO chains
197 since they possess an intrinsic consensus SUMOylation motif. SUMO2/3 chains can even be
198 capped with SUMO1 which leads to hybrid chain formation (Chang & Yeh 2020). Three SUMO
199 adducts above the unmodified wild type Zbtb21 was observed. While the lowest one represents
200 one SUMO molecule attached covalently with Zbtb21 (which is ~ 11 kD), the upper two should
201 represent SUMO chains with different lengths on Zbtb21.

202 Most ZBTB family members function as transcription repressor (Lee & Maeda 2012). Yet,
203 ZBTB14 displays an activation or repression effect on different promoters (Kaplan & Calame
204 1997), and SUMOylation of Zbtb14 turns it to be a potent transcription repressor on *pu.1*
205 promoter (Deng et al. 2022). Although it has been reported that ZBTB21 mostly contributes to
206 transcriptional repression (WangXuWangTianWang & Ji 2023), our results show that Zbtb21
207 also plays a dual role in transcription. While Zbtb21 can inhibit *CDC6* promoter, it activates
208 *zbtb14* promoter. Intriguingly, no matter activation or repression, SUMOylation of Zbtb21 can

209 enhance its transcriptional activities. Further studies on the function of Zbtb21 will be helpful to
210 better understand the bi-directional transcriptional regulation in which Zbtb21 is implicated.

211

212 **Conclusions**

213 ZBTB21 is a novel substrate of SUMOylation. The transcription activity of ZBTB21 is
214 affected by SUMO modification, which would be important for its function.

215

216 **Acknowledgments**

217 The authors are grateful to Y Chen (from Shanghai Jiao Tong University School of
218 Medicine, Shanghai, China) for technical support.

219

220 **References**

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261 reveals that ZBTB transcription factors contribute to chromatin interactions.
262

Figure 1

Zbtb21 is identified as a SUMOylated substrate.

(A) Western blot analyses (anti-HA) of HA-tagged wild type (WT), Zbtb21^{K419R}, Zbtb21^{K419R}, Zbtb21^{K845R}, and Zbtb21$^{K419/845R}$ mutant proteins expressed in HEK293T cells in the absence or presence with the SUMO conjugating enzyme UBC9 and SUMO1. (B) HA-tagged wild type (WT), Zbtb21^{K419R}, Zbtb21^{K419R}, Zbtb21^{K845R}, and Zbtb21$^{K419/845R}$ mutant proteins were immunoprecipitated with an anti-HA antibody from HEK293T cells co-expressing SUMO1, and SUMOylated Zbtb21 protein was detected by western blot with an anti-SUMO1 antibody.

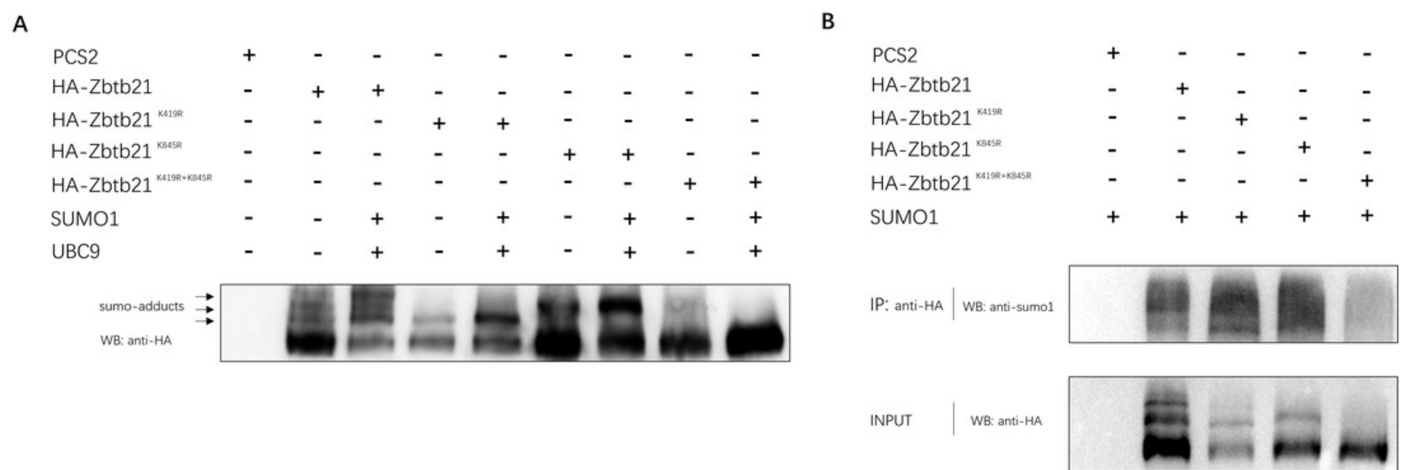

Figure 2

SUMOylation of Zbtb21 affects its transcriptional activity.

Luciferase reporter assays in HEK293T cells expressing HA-tagged wild type (WT), $Zbtb21^{K419R}$, $Zbtb21^{K845R}$, and $Zbtb21^{K419/845R}$ mutant plasmids on *CDC6* (A) and *zbtb14* reporters (B). Bars showed the relative luciferase activity (Student t test, N = 3. Error bars represent mean ± SEM. ns: not signifcant, *P < 0.1, **P < 0.01).

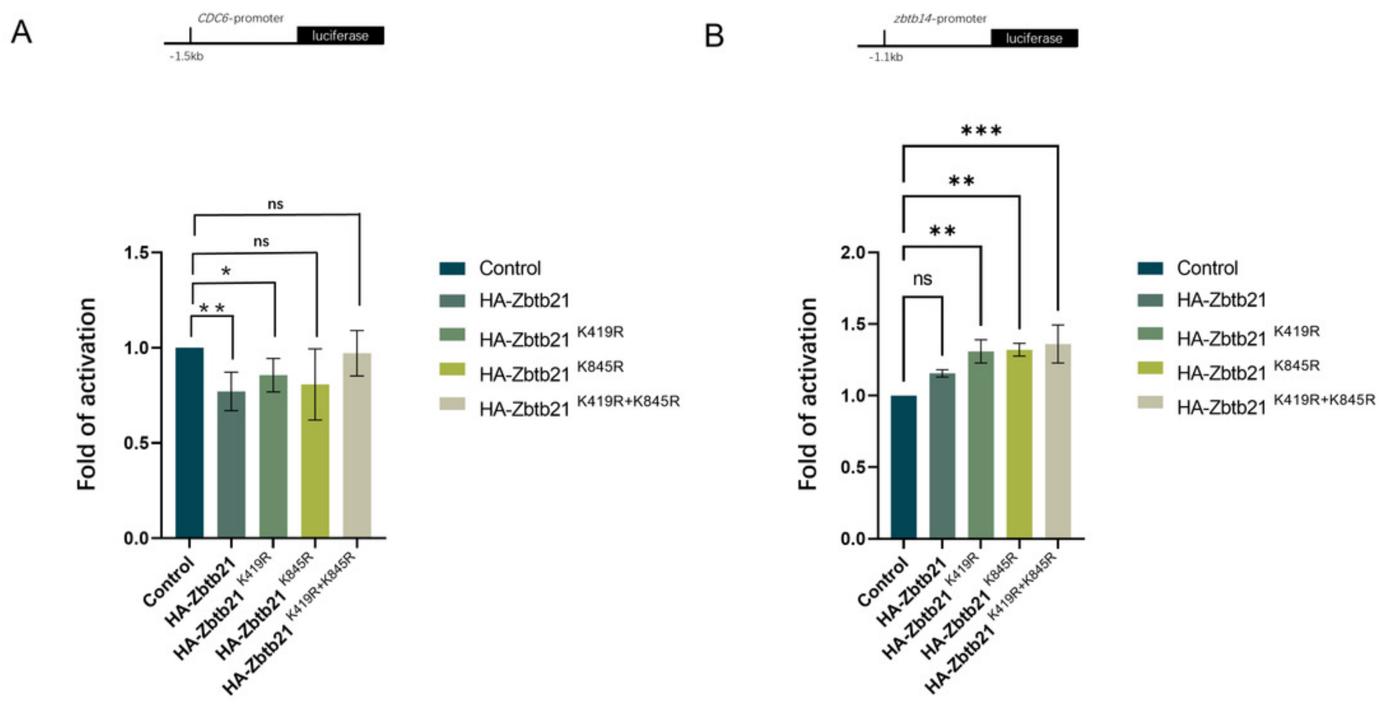

Figure 3

SUMOylation of Zbtb21 is irrelevant with its subcellular localization.

Immunofluorescence analyses of HEK293T cells expressing HA-tagged WT, Zbtb21^{K419R}, and Zbtb21^{K845R} mutant proteins. Cell nuclei were stained with DAPI.

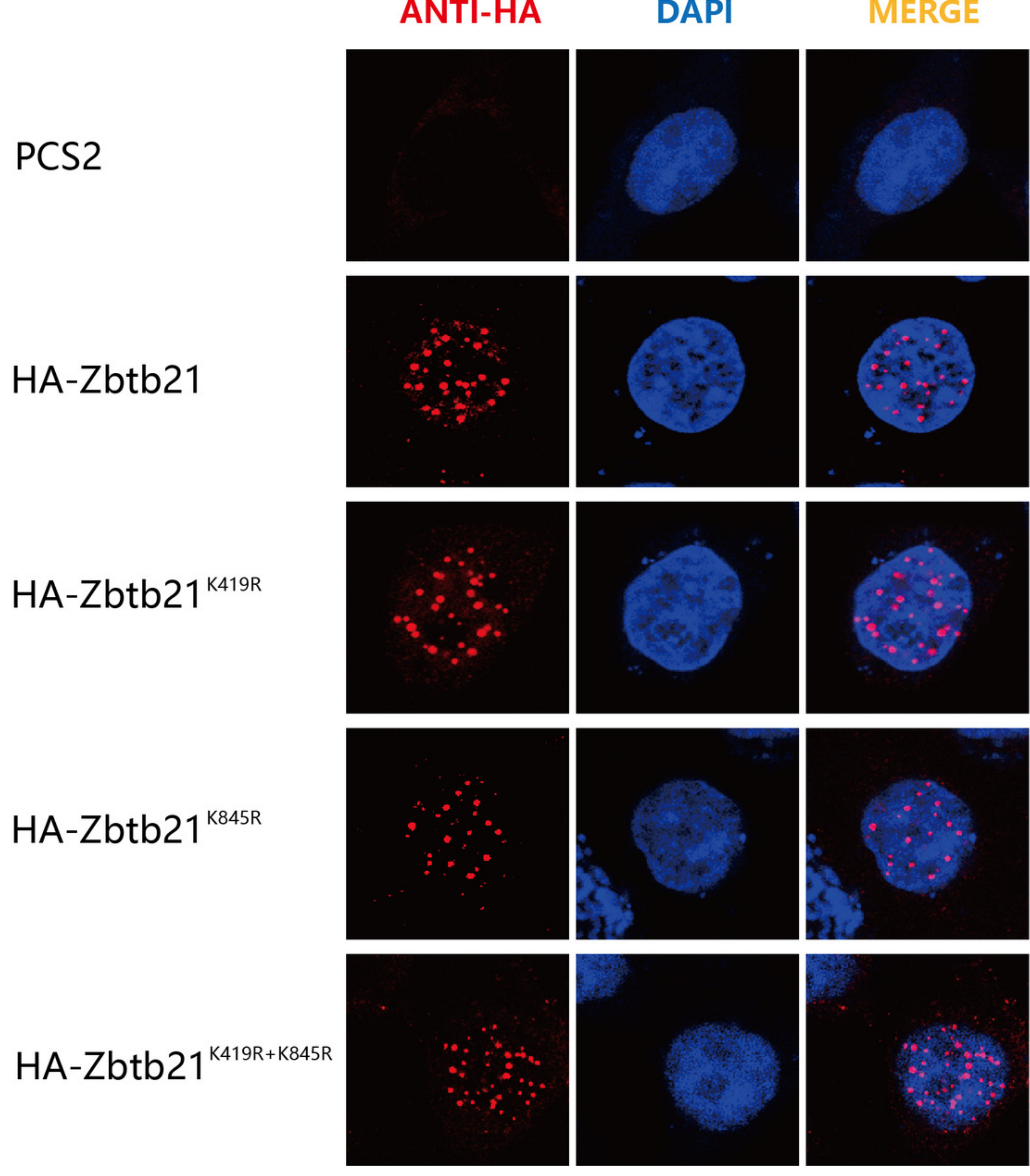
ANTI-HA
DAPI
MERGE
PCS2
HA-Zbtb21
HA-Zbtb21 K419R
HA-Zbtb21 K845R
HA-Zbtb21 K419R+K845R

Figure 4

SUMOylation of Zbtb21 does not affect the hetero-dimerization with Zbtb14 or homo-dimerization with Zbtb21.

(A) Co-IP experiments conducted in HEK293T cell transfected with HA-tagged Zbtb14 and Flag-tagged Zbtb21. (B) Co-IP experiments conducted in HEK293T cell transfected with HA-tagged Zbtb21 and Flag-tagged Zbtb21 constructs. Flag antibody was applied for immunoprecipitation and HA antibody was used for western blot analysis.

A

PCS2	+	-	-	-	-
HA-Zbtb21	-	+	-	-	-
HA-Zbtb21 K419R	-	-	+	-	-
HA-Zbtb21 K845R	-	-	-	+	-
HA-Zbtb21 K419R+K845R	-	-	-	-	+
FLAG-Zbtb14	+	+	+	+	+

IP: anti-Flag | WB: anti-HA

INPUT | WB: anti-FLAG

B

PCS2	+	-
HA-Zbtb21 K419R+K845R	-	+
FLAG-Zbtb21 K419R+K845R	+	+

IP: anti-FLAG | WB: anti-HA

INPUT | WB: anti-FLAG

Table 1(on next page)

Table 1

Primers used in this work.

1

	Primers for plasmid generation
zf zbtb21	Forward：5'-CCGGAATTCACGTCAAATACAAGATGGATG-3' Reverse：5'-CCGCTCGAGTTACGTATGGCTCTGTTCGTG-3'
zf zbtb21^{K419R}	Forward：5'-ACACAGGATTAGAGCAGAGCCCA-3' Reverse：5'-TGGGCTCTGCTCTAATCCTGTGT-3'
zf zbtb21^{K845R}	Forward：5'-GTTGCAGGTTAGAGAGGAACCTC-3' Reverse：5'-GAGGTTCCTCTCTAACCTGCAAC-3'
hs CDC6-promoter	Forward：5'-CGGACGCGTATTCGGATTTGGCGCGAGCG-3' Reverse：5'-CGGCCATGGGACGACAGCACAGCTAGATT-3'
zf zbtb14-promoter	Forward：5'-CGGGGTACCCATCAGTTGTATCTTAGGTACAG-3' Reverse：5'-CCGCTCGAGTGGACTCCTCATGTTTGCTCT-3'

2